mTORC1 drives HIF-1α and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3

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Abstract

Recent clinical trials using rapalogues in tuberous sclerosis complex (TSC) show regression in volume of typically vascularised tumours including angiomyolipomas (AMLs) and sub-ependymal giant cell astrocytomas (SEGAs). By blocking mechanistic/mammalian target of rapamycin complex 1 (mTORC1) signalling, rapalogue efficacy is likely to occur in part through suppression of hypoxia inducible factors (HIFs) and vascular endothelial growth factors (VEGFs). We show that rapamycin reduces HIF-1α protein levels, and to a lesser extent VEGF-A levels, in renal cystadenoma cells in a *Tsc2+/-* mouse model. However the precise mechanism(s) underpinning HIF-1α and VEGF-A regulation by mTORC1 are uncertain and better delineation of this angiogenic signalling pathway is fundamental for the development of therapeutic strategies for vascularised tumours. We establish that mTORC1 drives HIF-1α protein accumulation through enhanced transcription of HIF-1α mRNA, a process that is suppressed via inhibition of signal transducer and activation of transcription 3 (STAT3). We reveal that STAT3 is directly phosphorylated by mTORC1 on Ser727 during hypoxia, promoting HIF-1α mRNA transcription. mTORC1 also regulates HIF-1α synthesis on a translational level via co-operative regulation of both initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase-1 (S6K1), whilst HIF-1α degradation remains unaffected. We therefore propose that mTORC1 drives HIF-1α synthesis in a multi-faceted manner through 4E-BP1/eIF4E, S6K1 and STAT3. Interestingly, we observe a disconnect between HIF-1α protein levels and VEGF-A expression. While both S6K1 and 4E-BP1 regulate HIF-1α translation, VEGF-A is primarily under the control of 4E-BP1/eIF4E. S6K1 inhibition reduces HIF-1α but not VEGF-A expression, suggesting that mTORC1 mediates VEGF-A expression via both HIF-1α-dependent and -independent mechanisms. Our work has important implications for the treatment of vascularised tumours, where mTORC1 acts as a central mediator of STAT3, HIF-1α, VEGF-A and angiogenesis via multiple signalling mechanisms.

Introduction

Tumour vascularisation is regulated by hypoxia inducible factor (HIF) proteins. HIFs drive expression of over 800 different genes, many of which control cellular processes involved in cancer progression.[1] As well as promoting angiogenesis, HIF activation alters cellular metabolism resulting in enhanced glycolytic flux promoting survival under hypoxia.[2] Inappropriate activation of HIF-1α under normoxia causes a metabolic shift towards aerobic glycolysis, driving the Warburg effect which is characteristic of tumour cells.[3] HIFs also promote cell proliferation, survival and metastasis through secretion of growth factors,[4] motility and permeability factors[5] as well as expression of genes involved in the degradation/remodelling of the extracellular matrix.[6, 7]

HIF is centrally involved in the pathology of a number of inherited diseases where patients are predisposed to renal cell carcinoma (RCC) including Von Hippel-Lindau disease (VHL),[8] Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC),[9] and Birt-Hogg-Dubé (BHD) syndrome.[10] In VHL, loss of VHL protein function prevents ubiquitin-targeted proteasomal removal of HIF, resulting in inappropriate accumulation of HIF protein under normoxia.[11, 12] Likewise in HLRCC, HIF protein stability is enhanced via loss of fumarate hydratase enzyme and a build-up of intracellular fumarate.[9] In this context, it is known that aberrant accumulation of HIF protein drives RCC.

Tuberous Sclerosis Complex (TSC) is a genetic disorder that predisposes patients to non-malignant tumours in multiple organ systems. These include angiomyolipomas (AML) of the kidneys, cutaneous angiofibromas, subependymal nodules (SENs) or sub-ependymal giant cell astrocytomas (SEGAs) and cortical tubers in the brain, cardiac rhabdomyomas, lymphangioleiomyomatosis (LAM) in the lungs, and hamartomas in the eye (see review[13]). TSC-associated tumours are highly vascularised and express elevated levels of CD31, a marker of the vascular endothelium, and the angiogenic marker VEGF.[14] In TSC, the major factor driving angiogenesis is thought to be a result of increased signal transduction through Rheb and mammalian target of rapamycin complex 1 (mTORC1). Rheb/mTORC1 is normally repressed by the TSC:TBC tumour suppressor complex, that is functionally lost in TSC tumours.[15] Indeed, regression of tumour volume is apparent in TSC patients[16, 17] and mouse models[18] after mTORC1 inhibition. Angiogenesis inhibitors have also demonstrated therapeutic benefit and increased survival in TSC mouse models.[19] Given that the anti-angiogenic effects of rapamycin are well established in a variety

of diseases,[20-22] there is a surprising lack of clarity within the literature regarding the mechanisms behind mTORC1-mediated angiogenesis.

mTORC1 is comprised of the serine/threonine protein kinase mTOR, Lst8 and Raptor. Raptor associates with mTORC1 substrates through mTOR signalling (TOS) motifs to facilitate their recruitment and phosphorylation.[23] Inappropriate activation of mTORC1 has been reported in a wide variety of malignancies and mTOR inhibitors are currently licensed or undergoing clinical trials for several different cancer types.[24] We previously demonstrated that HIF-1α is modulated by mTORC1, with Rheb over-expression propagating high levels of HIF transcriptional activity in a rapamycin sensitive fashion.[25] Subsequent studies have supported this evidence, however there is a significant degree of conflict within the literature regarding the mechanism by which mTORC1 regulates HIF-1α. For example, Hudson *et al.* showed evidence that HIF-1α stability was reduced by rapamycin but HIF-1α translation and transcription were unaffected.[26] While Duvel *et al.* showed evidence that regulation of HIF-1α downstream of mTORC1 was primarily the result of increased 4E-BP1 phosphorylation and cap-dependent translation.[27] Tandon *et al.* also demonstrated that mTORC1 regulated the translation of HIF-1α, primarily via S6K1. Where shRNA mediated knockdown of S6K1 had comparable affects to rapamycin upon HIF-1α activity in PTEN deficient cells.[28] Active mTORC1 has also been reported to increase HIF-1α mRNA levels in cells devoid of TSC2[27, 29] indicative of transcriptional control. Conversely, a study using human SN12C renal cell carcinoma cells with and without VHL indicated that HIF-1α mRNA levels were insensitive to mTOR inhibition,[30] while Treins *et al.* found that mRNA levels of HIF-1α were not increased with insulin stimulation,[31] suggesting mTORC1 independence.

Whilst it is clear that mTORC1 plays a fundamental role in tumour angiogenesis, the mechanisms underpinning this are unclear and a complete analysis of these mechanisms is absent from the literature. Through various lines of investigation, we systematically challenge a series of potential mechanisms by which mTORC1 might regulate HIF-1α. By utilising an array of different assays to explore HIF-1α activity and mTORC1 signalling through S6K1, 4E-BP1, and STAT3, we provide evidence that underpins a better understanding of how mTORC1 regulates angiogenesis.

Results

In *Tsc2*+/- mice, rapamycin dramatically reduced mTORC1 signalling, HIF-1α and VEGF-A protein levels in renal cystadenoma cells

We previously demonstrated that HIF-1α activity was markedly elevated in *TSC2*-/- cell lines and sensitive to rapamycin inhibition.[25] We therefore examined the effects of rapamycin on the expression of HIF-1α and its gene target VEGF-A in a *Tsc2*+/- mice model (described here[32]) (Figure 1). These mice exhibit reduced TSC2 expression in cystadenomas as demonstrated in Figure 1A.

12 month old *Tsc2*+/- mice were treated with 10 mg/kg rapamycin 5 times a week (or vehicle as a control) for 2 months. Kidney sections were subjected to immunohistochemical (IHC) analysis to determine HIF-1α and VEGF-A protein levels (Figure 1B). Ribosomal protein S6 (rpS6) phosphorylation was also analysed as a readout of mTORC1 signalling. As predicted, strong IHC staining of HIF-1α and VEGF-A total protein as well as rpS6 phosphorylation was apparent in the cells lining and projecting into the lumen of the renal cyst, supporting the involvement of HIF-1α and VEGF-A in TSC pathology. Rapamycin potently reduced HIF-1α protein levels (as well as phosphorylation of rpS6), suggesting that the anti-tumourigenic effects of rapalogues are in part caused by repression of the mTORC1/HIF-1α angiogenic response. VEGF-A expression was also reduced by rapamycin, albeit to a lesser extent. Brugaralas *et al.* previously reported that VEGF-A expression was relatively insensitive to rapamycin in cell line models for TSC, we show that this effect also translates to mouse models, suggesting that rapalogues may not be fully effective at suppressing VEGF-A expression in TSC patients.[29]

HIF-1α is potently induced by active mTOR mutants

To explore this mechanism further, we employed cell line models allowing us to manipulate specific facets of mTORC1 signalling. We first utilised two constitutively active mTOR mutants; L1460P and E2419K (see Figure 2A) originally identified in yeast by Urano *et al.*[33] By expressing these active mTOR mutants alongside a luciferase reporter vector driven by HIF-1α response elements, we were able to specifically examine the effects of mTOR on HIF-1α. As Figure 2B demonstrates, we observed a 3-fold increase in HIF-1α activity in cells expressing active mutants compared to wild-type. As mTORC1 activity is dependent upon an amino acid input (for review see[34]); and HIF-1α regulation has not been studied in the context of amino acid availability, we utilised these mutants to examine the effects of nutrient withdrawal

upon HIF-1α. Indeed, nutrient withdrawal potently inhibited HIF-1α activity in cells expressing either wild-type or the E2419K mutant of mTOR. In concordance with our previous study, HIF-1α activity was also partially resistant to amino acid deprivation in cells expressing L1460P.[35] This result is therefore the first direct evidence that amino acids regulate HIF-1α activity via an mTORC1-dependent mechanism. Given that HIF-1α activity in cells expressing the L1460P mutant was partially insensitive to nutrient withdrawal, it is possible that the L1460P mutant is inappropriately targeted to the lysosomes and could therefore be a useful tool for investigating amino acid signalling to mTORC1. Whilst it is clear that mTORC1 mediates HIF-1α, it remains unclear how this regulation occurs, figure 2C indicates the possible mechanisms underlying this regulation.

Rapamycin does not affect the protein stability of HIF-1α

We next analysed the effects of rapamycin upon endogenous HIF-1α protein accumulation in HEK293 cells under hypoxia (Figure 3A). Cycloheximide, which blocks ribosomal translocation, was used as a control to prevent *de novo* protein synthesis of HIF-1α. As Figure 3A indicates, cyclohexamide pre-treatment is sufficient to completely ablate HIF-1α protein synthesis under hypoxia. As expected, rapamycin pre-treatment caused a reduction in HIF-1α protein levels by approximately one third at all time points (3A), clearly demonstrating that mTORC1 promotes accumulation of the HIF-1α protein. Hudson *et al.*'s work indicated that rapamycin decreased the stability of HIF-1α,[26] whereas other groups have indicated that rapamycin reduces the synthesis of HIF-1α on a transcriptional or translational level.[26-28] Given that we were able to completely block HIF-1α synthesis with cyclohexamide, a pulse-chase experiment was performed in hypoxic HEK293 cells in the presence and absence of rapamycin (Figure 3B) to determine whether HIF-1α stability is affected by rapamycin.

In the absence of *de novo* HIF-1α protein synthesis, HIF-1α levels rapidly decreased with a loss of approximately 50% of HIF-1α protein after just 5 min. This rapid deterioration of HIF-1α protein may suggest residual activity of prolyl-hydroxylase enzymes, even under hypoxia. Importantly, however, rapamycin did not impact the half-life of HIF-1α. Furthermore, inhibition of mTOR with the ATP-competitive inhibitor, KU-0063794, had no additional effect upon the stability of HIF-1α when directly compared to rapamycin (data not shown). Densitometry analysis of the three independent experiments

further supported this conclusion and is demonstrated in Figure 3C, no significant differences were observed between the rapamycin treated and pre-treated samples at any time-points analysed. Our work determines that mTORC1 promotes HIF-1α through increased synthesis rather than reduced degradation in support of the work by Duvel and Tandon *et al.*[27, 28]

Both rapamycin and KU-0063794 effectively suppress HIF-1α protein accumulation

We next analysed HIF-1α protein levels in hypoxic HEK293 cells expressing the active mutant of mTOR (E2419K). As predicted, the E2419K mutant induced a greater than 2-fold increase in endogenous HIF-1α protein levels after 2 h of hypoxia (Figure 4A), suggesting that during the initial stages of hypoxia, mTORC1 activation is crucial for promoting HIF-1α synthesis to elicit the hypoxic response. Phosphorylation of 4E-BP1 and rpS6 was analysed to demonstrate upregulation of mTORC1 in the presence of the active E2419K mutant. Interestingly, we observed fluctuations in 4E-BP1 Ser65 phosphorylation under hypoxia, this is likely to be the result of feedback inhibition to mTORC1 from the HIF-1α target REDD1.[36] rpS6 phosphorylation and HIF-1α protein levels were maintained indicating that mTORC1 is not fully suppressed.

Previous work demonstrates that some substrates of mTORC1 are more robustly inhibited with mTOR kinase inhibitors as opposed to rapalogues.[37, 38] To determine whether mTOR kinase inhibitors may be more effective than rapamycin at suppressing HIF-1α, we compared the effects of rapamycin to the ATP-competitive KU-0063794 mTOR kinase inhibitor (Figure 4B).[39] Treatment with KU-0063794 suppressed Akt phosphorylation at the mTORC2 site (Ser473), as well as reducing rpS6 phosphorylation, indicating suppression of both mTOR complexes. Ser65 phosphorylation of 4E-BP1 was partially rapamycin insensitive, as has previously been reported.[40] Interestingly, we observed complete recovery of 4E-BP1 phosphorylation during rapamycin treatment after just 2 h. Conversely, Choo *et al.* indicated that this recovery took approximately 6 h, it is therefore likely that over-expression of the active mTOR mutant within these cells caused a faster recovery from rapamycin repression.

mTORC1 inhibition with rapamycin caused a significant suppression in HIF-1α activity as before, however, no further inhibition of HIF-1α protein levels was observed with KU-0063794 compared to rapamycin. This confirms that mTORC1, rather than mTORC2 is driving the accumulation of HIF-1α

protein. Also consistent with previous observations, rapamycin treatment caused a slight increase in Akt phosphorylation, which is likely to be a result of reduced negative feedback signalling to IRS-1 via S6K1.[41]

Active S6K1 promotes HIF-1α protein accumulation

As depicted in Figure 2C, mTORC1 may promote HIF-1α synthesis via enhanced protein translation. S6K1 promotes protein translation through phosphorylation of numerous eukaryotic initiation factors[42-44] and may drive HIF-1α protein synthesis in this manner. Tandon *et al.* reported that S6K1 was essential for the translation of HIF-1α,[28] whereas Duvel *et al.* showed that S6K1 knockdown had no effect on a translational luciferase reporter driven by the HIF 5'UTR.[27]

To address this conflict and assess the involvement of S6K1 in HIF-1α regulation, we utilised a constitutively active S6K1 mutant (F5A-E389-R3A see Figure 5A). F5A-E389-R3A contains a mutation within the mTORC1 signalling (TOS) motif, a triple mutation to the 'RSPRR' motif which is acts as an mTORC1-mediated auto-inhibitory domain, as well as an E389 phospho-mimetic mutation within the linker region of S6K1. This F5A-E389-R3A mutant therefore drives a constitutively high level of S6K1 activity in cells regardless of mTORC1 activity.[45] HEK293 cells were transfected with either pRK7 or HA-S6K1-F5A-E389-R3A and cultured under hypoxia, HIF-1α protein accumulation was determined over a 4 h time-course. Expression of the active S6K1 mutant caused an approximate 2-fold increase in HIF-1α protein when compared to empty vector (Figure 5B). Conversely, the impact upon VEGF-A expression was not as dramatic, with the active S6K1 mutant causing only a slight increase in protein levels of VEGF-A. To confirm this effect, HEK293 cells were treated for 1 h prior to hypoxic incubation with the highly specific S6K1 inhibitor, PF-4708671.[46] As Figure 5D demonstrates, HIF-1α protein levels were highly sensitive to S6K1 inhibition, however VEGF-A expression levels were maintained despite the drop in HIF-1α protein. This suggests that VEGF-A expression is mediated via both HIF-1α-dependent and -independent mechanisms, as has previously been reported within the literature.[47]

In order to determine whether S6K1 modulated HIF-1α expression on a transcriptional and/or translational level, HIF-1α mRNA levels were also examined. Figure 5D demonstrates that HIF-1α mRNA levels are unaffected by either expression of the active S6K1 mutant or inhibition of S6K1 with PF-

4708671, indicating that S6K1 mediates HIF-1α protein expression solely through protein translation. Interestingly, PF-4708671 acted as a dominant inhibitor of HIF-1α, reducing HIF-1α protein expression to a level lower than cells expressing empty vector, even in the absence of insulin stimulation. PF-4708671 also caused an increase in S6K1 Thr389 phosphorylation, as previously reported.[46]

eIF4E availability is a rate limiting factor in the synthesis of both HIF-1α and VEGF-A

We next assessed 4E-BP1's role as a negative regulator of HIF-1α through inhibition of eIF4E. eIF4E availability is considered a rate-limiting factor of 5'-cap-dependent translation downstream of mTORC1.[48] Duvel *et al.* showed evidence that 4E-BP1 represses HIF-1α by acting as a negative regulator of eIF4E,[27] in contrast to Hudson *et al.* who observed no effects on HIF-1α translation with rapamycin treatment.[26] To examine whether inhibition of eIF4E could modulate HIF-1α activity, *TSC2*-/- MEFs, which exhibit constitutively active mTORC1 signalling, were transfected with the HIF-1α luciferase reporter alongside mutant or wild-type 4E-BP1 and cultured under hypoxia. Two mutants of 4E-BP1 were utilised; 4E-BP1(F114A), which contains a mutation within the TOS motif (so cannot be phosphorylated by mTORC1 and dominantly inhibits eIF4E), and the Y54A/L59A mutant which contains mutations within the eIF4E-binding domain. The 4E-BP1 Y54A/L59A mutant serves as a negative control as it is unable to interact with eIF4E. As expected; 4E-BP1(F114A) was less phosphorylated and consequently bound more avidly to eIF4E when compared to wild-type 4E-BP1 (Figure 6A). The 4E-BP1(F114A) TOS mutant, which potently bound to eIF4E, repressed the activity of HIF-1α by approximately one third compared with the wild-type (35 ±2%). In contrast, both the Y54A/L59A mutant and wild-type 4E-BP1 had minimal effects upon HIF-1α activity. The 4E-BP1(F114A) TOS-mutant was also able to suppress HIF-1α protein accumulation in the HEK293 cells, as demonstrated by the hypoxic time course in Figure 6C. There was an approximate 50% reduction in HIF-1α protein levels after 2 h of hypoxia in cells expressing 4E-BP1(F114A). This result implies that mTORC1 mediated phosphorylation of 4E-BP1 is a critical factor driving HIF-1α protein synthesis. In contrast to the effects of S6K1 inhibition, expression of the 4E-BP1-F114A mutant caused a significant reduction in VEGF-A expression (Figure 6C), indicating that repression of 4E-BP1 by mTORC1 is crucial for the VEGF-A response. HIF-1α mRNA levels were unaffected by expression of the 4E-BP1-F114A mutant (Figure 6D), suggesting that, in a manner

analogous to S6K1, this regulation is also on a translational level. This data suggests that mTORC1 mediates HIF-1α activity by promoting 5'-cap-dependent translation through both 4E-BP1/eIF4E and S6K1, while the translation of VEGF-A is primarily regulated by 4E-BP1/eIF4E and not S6K1.

mTORC1 drives the transcription of HIF-1α via STAT3

To further investigate mTORC1 involvement in HIF-1α mRNA transcription, HEK293 cells were transfected with the active mTOR(E2491K) mutant and insulin stimulated (to promote maximal activation of mTORC1) in the presence or absence of rapamycin. Q-PCR analysis revealed a substantial rapamycin sensitive increase in HIF-1α mRNA levels in response to insulin, indicating a transcriptional facet of control through mTORC1 (Figure 7A). Although elevation of HIF-1α mRNA levels has been previously reported within TSC2-/- MEFs,[27, 29] the mechanism driving HIF-1α mRNA transcription downstream of mTORC1 is unknown. We hypothesised that another downstream signalling target of mTORC1 may be involved in this regulation. Previous work in tumour cells reported links between HIF-1α and STAT3.[49] Furthermore, Yokogami *et al.* suggested a link between mTORC1 and STAT3, demonstrating phosphorylation of a C-terminal STAT3 peptide using a crude *in vitro* mTOR kinase assay.[50] Other studies have shown upregulation of STAT3 signalling in cell line models for TSC,[51, 52] however the direct phosphorylation of STAT3 by mTORC1 has yet to be confirmed. The mTOR kinase assay employed by Yokogami *et al.* was not optimal; for instance, a short STAT3 peptide (which did not contain any potential Raptor interacting motifs for substrate recognition) was assayed alongside over-expressed mTOR in the absence of Raptor, using $MnCl_2$ and high ATP levels to artificially enhance kinase activity. We therefore utilised our previously optimised mTORC1 specific kinase assay,[35] with more physiologically relevant assay conditions to verify whether full length recombinant STAT3 was indeed a direct substrate for mTORC1 (7B). As Figure 7B shows; we observe robust phosphorylation of STAT3 at Ser727 by mTORC1. Inclusion of purified recombinant GTP-loaded GST-Rheb, a potent and specific activator of mTORC1, increased Ser727 phosphorylation of STAT3 in a manner similar to the well characterised mTORC1 substrate; 4E-BP1. This data confirms that STAT3 is a direct mTORC1 target.

To determine whether STAT3 was involved in HIF-1α mRNA expression, HEK293 cells were pre-treated with either rapamycin, or STAT3 inhibitors: FLLL31 (an upstream JAK2 inhibitor) and 5,15-

diphenylporphyrin (5,15,DPP), an Src homology-2 domain (SH2) antagonist (Figure 7D) in the presence of insulin. CNTF (ciliary neurotrophic factor) was also utilised to stimulate STAT3 Tyr705 phosphorylation as this is considered to be a priming event required for Ser727 phosphorylation by mTORC1.[53] In a manner analogous to rapamycin treatment, inhibition of STAT3 potently blocked insulin induced HIF-1α mRNA induction. This result suggests that the mechanism by which mTORC1 promotes HIF-1α mRNA expression is via phosphorylation of STAT3 at Ser727. HIF-1α protein levels were also examined to verify this finding. As Figure 7D indicates, both STAT3 inhibitors suppressed HIF-1α protein levels, further supporting the conclusion that STAT3 drives HIF-1α transcription. Intriguingly, the curcumin analog, FLLL31, had a dramatic dominant inhibitory effect on HIF-1α, reducing expression levels by approximately 80%. In contrast, both rapamycin and 5,15,DPP treatment both resulted in an approximate 30% reduction in HIF-1α expression. Analysis of rpS6, 4E-BP1 and STAT3 Tyr705 phosphorylation in these cells revealed that this was due to dual suppression of both JAK/STAT and mTORC1 signalling by FLLL31. This dual repression of both signalling pathways makes FLLL31 an ideal potential therapeutic for the treatment of vascularised tumours where mTORC1 is active.

Discussion

Although mTORC1 is known to potently activate HIF-1α,[25] the mechanism(s) governing this regulation are poorly defined. We designed this study to systematically examine specific facets of mTORC1 mediated angiogenesis to better understand this process. Although previous studies have suggested roles for either S6K1 or 4E-BP1 in driving HIF-1α activity, few studies have investigated the effects of both. We have used a variety of methodologies to characterise angiogenic signalling downstream of mTORC1 and show for the first time that both 4E-BP1 and S6K1 promote the translation of HIF-1α.

Furthermore, we confirm previous reports that mTORC1 drives HIF-1α translation and uncover the mechanism by which this occurs. We show that mTORC1 promotes the phosphorylation of STAT3 at Ser727 during hypoxia to promote HIF-1α transcription, and that STAT3 inhibition effectively represses insulin induced HIF-1α transcription in a similar manner to rapamycin.

Previous studies identified putative hypoxia response elements (HREs) in the HIF-1α promoter, suggesting an auto-regulatory loop whereby HIF-1α may up-regulate synthesis of its own mRNA.[54] This is

unlikely as expression of the S6K1 and 4E-BP1 mutants caused substantial effects on HIF-1α protein but not mRNA levels. We show that HIF-1α mRNA levels are suppressed with mTORC1/STAT3 inhibition and conclude that mTORC1 mediates HIF-1α transcription via STAT3, confirming STAT3 as direct substrate for mTORC1. Like HIF-1α; STAT3 mediates several cellular processes linked with cancer progression including cellular survival, proliferation and metastasis.[55, 56] It is likely that upregulation of STAT3 contributes to the pathology of TSC-associated hamartomas and cancer progression in tumours where mTORC1 is active.

Previous studies demonstrated upregulation of STAT3 Tyr705 phosphorylation in mouse/cell line models for TSC.[51, 52] Conversely, our results indicate that Tyr705 phosphorylation is insensitive to rapamycin inhibition. This may suggest that STAT3 Tyr705 phosphorylation is upregulated as a result of disrupted feedback mechanisms due to prolonged mTORC1 activation in the TSC models.

Importantly, we demonstrate that the STAT3 inhibitor FLLL31 has significant inhibitory effects on HIF-1α transcription and translation, through dual suppression of both mTORC1 and STAT3 signalling pathways. FLLL31 may therefore be a more appealing drug for the treatment of vascularised tumours, with greater therapeutic benefit than mTORC1 or STAT3 inhibitors alone.

It is well documented that VHL mediates proteasomal degradation of HIF-1α under normoxia.[11] However, VHL and oxygen independent mechanisms of HIF-1α degradation have also been reported.[57] Hudson *et al.* showed evidence that rapamycin influences the stability of HIF-1α, while the transcription and translation are unaffected.[26] We show contrasting evidence in multiple experiments (Figures 5, 6 and 7) that both HIF-1α translation and transcription are subject to modification by mTORC1, in concordance with other studies examining HIF-1α synthesis.[27, 31, 58] Furthermore, we observe no difference in the half-life of HIF-1α protein in rapamycin treated and untreated cells. We therefore conclude that mTORC1 regulates the synthesis of HIF-1α (via both mRNA transcription and protein translation) whilst VHL mediates HIF-1α protein turnover (see figure 8).

S6K1 regulates a number of translation initiation factors, including eIF3,[42] eIF4B,[59] and elongation factor 2 kinase (eEF2k)[60] and may enhance HIF-1α translation via these substrates. The specific S6K1 inhibitor had a dramatic impact on HIF-1α protein levels whilst HIF-1α mRNA levels were maintained; indicating that S6K1 promotes HIF-1α translation but not its transcription. Interestingly, VEGF-A levels

were insensitive to inhibition or activation of S6K1, while expression of the dominant inhibitory 4E-BP1 mutant had a dramatic impact upon VEGF-A levels. This indicates that mTORC1/4E-BP1 signalling drives VEGF-A expression via both HIF-1α-dependent and -independent mechanisms. Given that rapamycin is known to cause incomplete suppression of eIF4E,[40] this finding explains why VEGF-A levels in the *Tsc2*-/+ mice are less sensitive to rapamycin inhibition than HIF-1α and phospho-rpS6 levels. Our data suggests that in the treatment of vascularised tumours, targeting eIF4E in combination with rapamycin may be a more effective strategy for reducing VEGF-A expression.

Previous work by Thomas *et al.* suggested that HIF-1α may be preferentially translated downstream of mTORC1 as a TOP (tract of pyrimidine) mRNA.[30] Two recent studies used ribosomal profiling to produce a unified model for 5'-TOP mRNA translation. These studies demonstrated that mTORC1 promotes the translation of TOP mRNAs via 4E-BP1 phosphorylation and eIF4E availability.[61, 62] Given that we observed S6K1 involvement in HIF-1α activity, as well as an mTORC1-dependent STAT3 input into HIF-1α mRNA transcription, it is likely that the rate of HIF-1α translation is proportional to the abundance of HIF-1α mRNA, rather than any selective qualities conferred by its mRNA structure. This is in concordance with current thinking that HIF-1α is not a genuine 5'-TOP mRNA.[63]

Our findings suggest that STAT3, 4E-BP1, and S6K1 act in concert to drive the angiogenic response through mTORC1. Placing mTORC1 as a central mediator of the hypoxic response via multiple signalling outputs; Figure 8 indicates the different mechanisms delineated within this study. Recent evidence has suggested that mRNAs translated by mTORC1 are regulated via eIF4E availability, and can be stratified into functional groups controlling oncogenic processes including cell proliferation, metabolism and invasion.[62] We show evidence that not all the oncogenic signalling outputs downstream of mTORC1 are mediated solely by eIF4E availability, and that both S6K1 and 4E-BP1 facilitate the angiogenic response at a translational level.

Materials and Methods

Animal treatment and Immunohistochemistry (IHC)

Animal procedures were performed in accordance with the UK Home Office guidelines and approved by the Ethical Review Group of Cardiff University. *Tsc2*+/- mice (described here[32]) were treated with 10

mg/kg rapamycin/vehicle via intra-peritoneal injection 5 times a week for 2 months. Animals were humanely killed and kidney sections prepared for IHC analysis. Antibodies against HIF-1α, VEGF-A and phosphorylated rpS6 (S6 p-S235/236) (Cell Signalling Technology, Danvers, USA) were used for IHC. SignalStain Boost Rabbit specific IHC Detection Reagent (Cell Signalling Technology) was used for antigen staining. Briefly, paraffin-embedded mouse kidney sections were deparaffinised and rehydrated. Sections were boiled for 10 min in 10 mM sodium citrate buffer (pH 6.0). After 3 washes in TBST, sections were blocked in 1.5% (v/v) goat serum for 10 min at room temperature. Antigen staining was performed according to manufacturer's protocol.

Plasmid details

Myc-mTOR and active mutant constructs,[35] HA-S6K1 constructs[45] and 4E-BP1 constructs[68] are described previously. The HIF-1α specific inducible luciferase reporter construct was purchased from Affymetrix Inc. (Vignate-Milano, Italy). Gateway recombination cloning technology (Life Technologies) was utilised to generate GST-STAT3 from an I.M.A.G.E. clone (purchased from ATCC, Manassas), in accordance with manufacturer's guidelines.

Antibodies and biochemicals

Anti-myc, anti-rpS6 phospho-Ser235/236, anti-eIF4E, anti-4E-BP1 phospho-Thr70, anti-STAT3 Ser727 and Tyr705, and anti-β-actin antibodies were obtained from Cell Signalling Technology. Anti-Rheb was obtained from Santa Cruz (Heidelberg, Germany), anti-HA was obtained from Roche (Hertfordshire, UK), and Anti-HIF-1α was obtained from BD transduction laboratories (Oxford, UK). Rapamycin was obtained from Calbiochem (Nottingham, UK). Dimethyloxallyl Glycine (DMOG) was purchased from R & D systems (Minneapolis, USA). KU-0063794 was purchased from Chemquest Ltd. (Cheshire, UK). Cycloheximide, insulin, PF-4708671, 5,15-DPP, FLLL31 and all other general lab chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Cell Culture and transfection

HEK293s, *TSC2*-/- and *TSC2*+/+ MEFs (a kind gift from Prof. D. Kwiatkowski, Harvard University, Boston) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal calf serum and 100 µ/ml penicillin streptomycin (Life Technologies). Transfections were carried out using Lipofectamine 2000 in accordance with manufacturer's protocol (Life Technologies).

Time course experiments

HEK293 cells were transfected, drug treated as indicated and placed into a Binder CB150 hypoxic chamber set at 1 % O_2 for the indicated time points. Cells were lysed directly in sample buffer (62.5 mM Tris HCL, 50 mM DTT, 2 % SDS (w/v), 10 % Glycerol (w/v), 0.1 % Bromophenol blue (w/v) pH 7.6) and sonicated for 5 x 20 s cycles on full power (30 amplitude microns) before analysis by western blotting.

Western Blotting

Lysates were resolved by SDS-PAGE and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Edinburgh, UK). Membranes were blocked in 5 % (w/v) dry milk powder dissolved in Tris-buffered saline containing 0.1 % (v/v) Tween, then probed with primary antibody and horse radish peroxidase-conjugated secondary antibody (Sigma-Aldrich). Proteins were visualized using Enhanced Chemiluminescent solution and Hyperfilm (both GE Healthcare, Buckinghamshire, UK). All western blots are representative of at least three independent experiments. Where densitometry analysis is shown, imageJ 1.43v software was utilised to determine relative levels of HIF-1α, standardising the untreated control sample to 1 after either 1 or 2 h in hypoxia.

Luciferase Assays

These assays were carried out as described previously,[35, 69] using the HIF-1α specific luciferase reporter construct (Affymetrix Inc.) and Promega dual-luciferase reporter assay system in accordance with manufacturer's protocol (Promega, Southampton, UK).

mTORC1 kinase assays

Kinase assays were carried out as previously described.[35] Recombinant GST-STAT3 was purified from rapamycin treated HEK293 cells and assayed in the presence and absence of 75 ng purified GST-Rheb for 1 h at 30^{o}C with gentle agitation.

Quantitative PCR

Q-PCR was performed as previously described.[10] Primer sets for HIF-1α (cat no. QT01039542) and β-actin (cat no. QT01680476) were purchased from Qiagen who retain the right to withhold primer sequence information. Fold change was calculated using the ddCT (delta-delta-Ct) method, standardised to β-actin. A dissociation step was performed to verify a single product was produced with each primer set. The size of PCR products was also verified by resolution on a 2% polyacrylamide gel with both HIF-1α and β-actin giving an approximate amplicon length of 104 bp. All Q-PCR primer assays in this study were at least 96% efficient.

Conflict of interest

The authors declare no conflict of interest.

Figure Legends

Figure 1: In *Tsc2*+/- mice, rapamycin reduced mTORC1 signalling, HIF-1α and VEGF protein levels in renal cystadenoma cells lacking functional Tsc2

A: Paraffin-embedded kidney sections from five 12 month old *Tsc2*$^{+/-}$ mice were analysed by IHC. Thirty five cystadenomas were analysed for Tsc2 expression. Tsc2 expression was either undetectable or markedly reduced within these lesions. **B:** *Tsc2*$^{+/-}$ mice of 12 months old were treated with 10 mg/kg rapamycin (n=4) or vehicle (n=4) for two months. Paraffin-embedded kidney sections were stained using IHC with an antibody against pS6 (Ser235/236), HIF-1α or VEGF-A. Thirty renal lesions were scored for each treatment group. All lesions from mice treated with rapamycin showed a significant reduction in phosphor-rpS6 and HIF-1α. VEGF was also reduced by rapamycin treatment although to a lesser extent.

Figure 2: HIF-1α is potently induced by active mTOR mutants.

A: mTOR schematic indicating conserved regions and mutation positions. Domains: FRB refers to FKBP12/Rapamycin binding domain, NRD: Negative regulatory domain. **B:** HEK293 cells transfected with the mTOR mutants as indicated in conjunction with the HIF-1α inducible luciferase reporter. Cells were starved of amino acids (AA) and cultured in media containing 1 mM DMOG (to mimic hypoxia) for 4 h. Cells were stimulated with 10 µg/ml insulin 15 min prior to lysis. Luminescence was measured and standardised to total protein as determined by a Bradford assay. Error bars indicate standard deviation between three independent experiments. **C:** mTORC1 may be able to regulate the synthesis, degradation or activity of HIF-1α. There are several different ways in which this may occur; dotted lines indicate potential mechanisms of regulation whilst solid lines indicate known facets of the pathway.

Figure 3: Rapamycin does not affect the protein stability of HIF-1α

A: HEK293 cells were treated with vehicle, 10 µg/ml cyclohexamide or 50 nM rapamycin for 30 min prior to hypoxic incubation for the given time points. **B:** HEK293 cells were cultured under hypoxia for 2 h to allow build-up of HIF-1α protein then pre-treated with 50 nM rapamycin or vehicle for 30 min. 10 µg/ml cycloheximide was added to the cells before hypoxic incubation for the given time points. Lysates were sonicated and analysed for HIF-1α and β-actin protein levels using western blotting. **C:** HIF-1α protein

levels from Figure 3B were analysed using densitometry. Data was collated from three individually repeated experiments and analysed for statistical significance. No difference in the half-life of HIF-1α was observed between the rapamycin treated and untreated samples.

Figure 4: Both rapamycin and KU-0063794 effectively suppress HIF-1α protein accumulation

A: HEK293 cells were transfected with wild-type or the active mutant of mTOR (E2419K) then placed into the hypoxic chamber for the given time points. Lysates were sonicated and analysed for HIF-1α protein levels, as well as phosphorylated and total 4E-BP1 and rpS6 to indicate mTORC1 activity. Result is representative of three independent replicated experiments. **B:** HEK293 cells expressing the active mutant were treated with 50 nM rapamycin (rap) or 1 μM KU-0063794 (KU) for 1 h prior to hypoxic exposure and a time course was performed as described in 4A. Akt phosphorylation was used as a readout for mTORC2 activity whilst phospho-rpS6 and phospho-4E-BP1 were used as indicators of mTORC1 activity.

Figure 5: Active S6K1 promotes HIF-1α protein accumulation

A: Schematic showing mutant constructs of S6K1 utilised, indicating conserved domains and mutated regions. **B:** HEK293s were transfected with either pRK7 or HA-S6K1-F5A-R3A-E389 and subjected to hypoxia for the given time points prior to lysis. HIF-1α protein levels were quantified using western blotting and imageJ software for densitometry analysis. Result is representative of three independent experiments. **C:** Untransfected HEK293 cells were treated for 1 h with 10 μM PF-4708671 and 15 min with 10 μg/ml insulin prior to 4 h hypoxic/normoxic incubation. Cells were lysed directly in sample buffer, sonicated and analysed for protein levels using western blotting. Densitometry analysis was performed using imageJ software. **D:** Duplicate HEK293 cells expressing either empty vector, wild-type of active S6K1 mutant were treated with vehicle or 10 μM PF-4708671 as indicated and incubated in the hypoxic chamber for 1 hr. mRNA was extracted from half the lysates and Q-PCR was performed to determine HIF-1α mRNA levels, standardised to β-actin. The remaining lysates were sonicated and analysed by western blotting for HIF-1α expression and S6K1 activity.

Figure 6: eIF4E availability is a rate limiting factor in the synthesis of HIF-1α and VEGF

A & B: *TSC2-/- MEFs* were transfected with either pACATG-2 or mutant constructs of 4E-BP1 alongside the HIF-1α luciferase reporter. Lysates were analysed for HIF-1α induced luciferase activity (standardised to total protein levels). Graph indicates results from three independent experiments. eIF4E was immunoprecipitated from the remainder of the lysates cells using m^7GTP beads and 4E-BP1 binding was assessed using western blotting. **C**: The TOS-mutant of 4E-BP1 was over-expressed in HEK293 cells cultured under hypoxia for the given time points. Lysates were analysed for total HIF-1α and VEGF levels using western blotting. **D:** HEK293 cells expressing empty vector/4E-BP1 mutant were cultured under hypoxia for 4 h, mRNA was extracted from the total lysates and Q-PCR was performed to determine HIF-1α mRNA levels (standardised to β-actin).

Figure 7: mTORC1 drives the transcription of HIF-1α via STAT3

A: HEK293 cells expressing empty vector or the active mutant of mTOR were cultured under hypoxia in the presence of insulin and or rapamycin as indicated. mRNA was extracted from total lysates, HIF-1α mRNA was quantified as standardised to β-actin. **B:** mTORC1 kinase assay: An active mTORC1 complex was purified from insulin stimulated HEK293 cells grown under serum-starved conditions as described, [35] GST-STAT3 and GST-4E-BP1 were purified from serum-starved HEK293 cells, as was GST-Rheb, which was then loaded with GTPγS. Purified mTORC1 complex was incubated alongside potential substrates with and without GTPγS-Rheb (as indicated) for 1 h at 30°C with gentle agitation. SDS-PAGE and western blotting with phospho-specific antibodies was utilised to determine specific phosphorylation events as well as mTOR/Raptor purification. **C:** Untransfected HEK293 cells were treated with rapamycin/STAT3 inhibitors for 45 min prior to 30 min of insulin/CNTF stimulation (as indicated). mRNA was extracted from total lysates and quantified as above (7A). **D:** HEK293 cells were pre-treated with STAT3 inhibitors 5 nM FLLL31, 50 nM 5,15,DPP or vehicle for 30 mins, then stimulated with 25 ng/ml CNTF (to stimulate STAT3 Tyr705 phosphorylation) and insulin (to stimulate mTORC1 activation) before 1 h incubation under hypoxia. Lysates were sonicated and analysed using western blotting. ImageJ software was utilised to analyse HIF-1α protein levels via densitometry.

Figure 8: mTORC1 mediated angiogenesis

Schematic showing multiple mTORC1 inputs into angiogenesis. As demonstrated in this study, mTORC1 regulates the translation of HIF-1α via both 4E-BP1/eIF4E and S6K1. Although S6K1 can also promote

HIF-1α translation, VEGF-A does not appear to be directly affected by active S6K1. mTORC1 also controls the transcription of HIF-1α mRNA in a rapamycin sensitive fashion via STAT3 phosphorylation, indicating that mTORC1 mediates angiogenesis via three distinct mechanisms.

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Figure 1

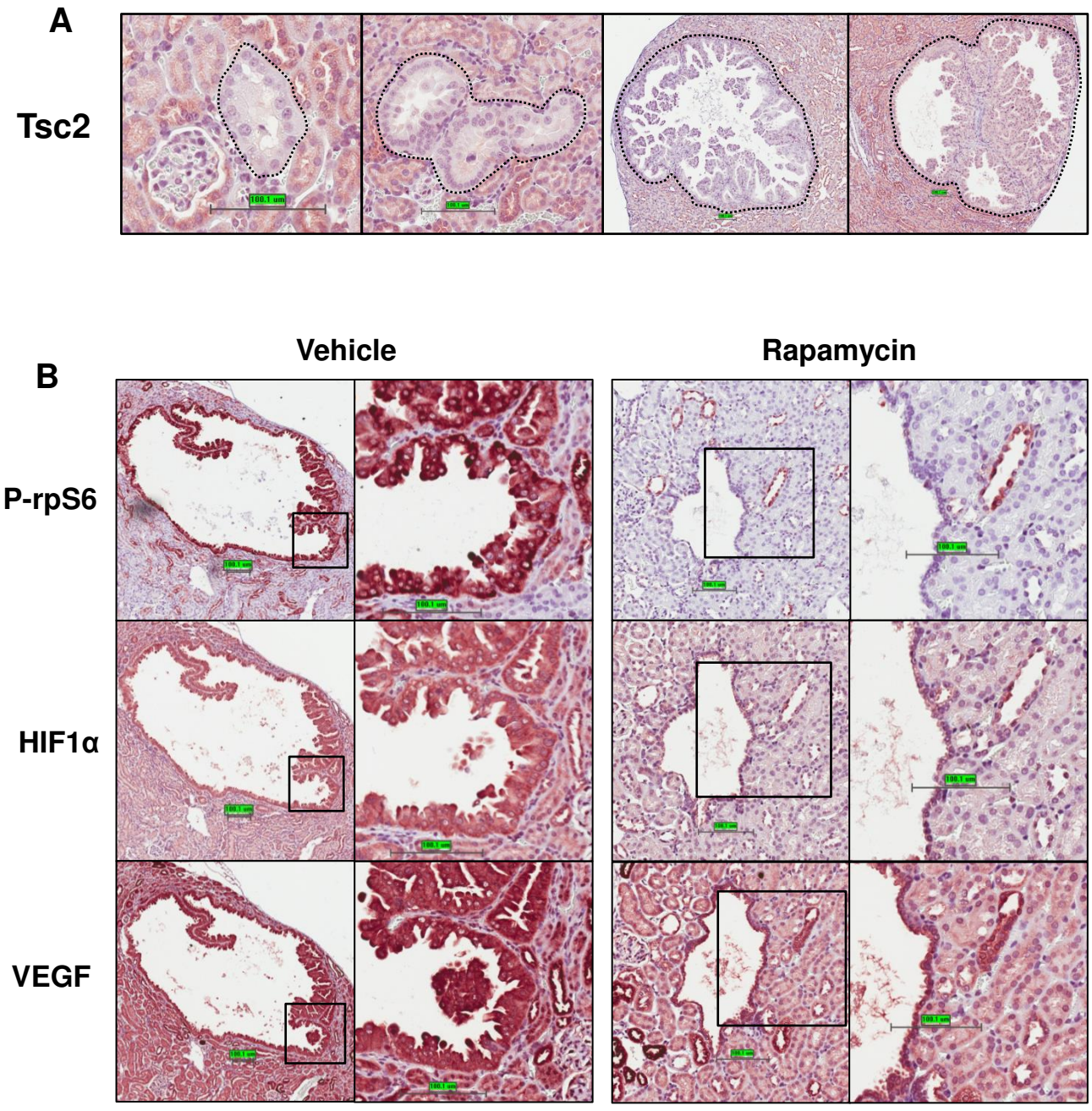
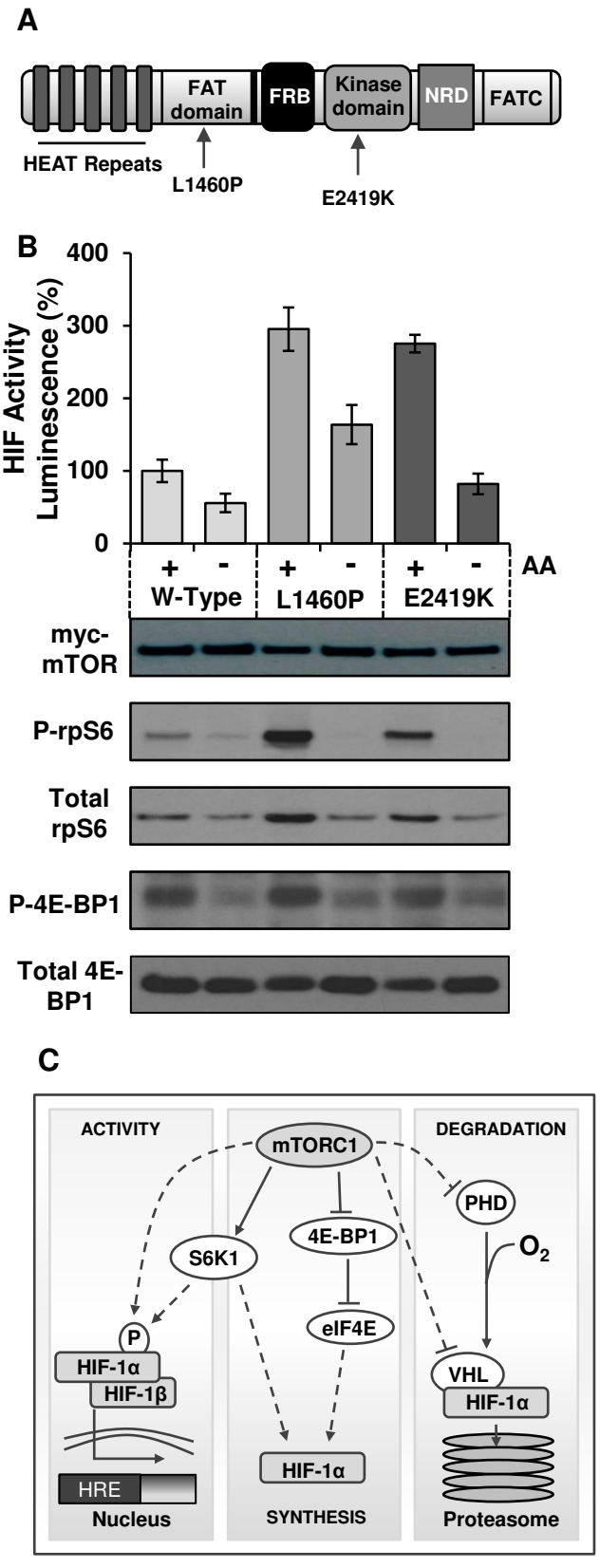


Figure 2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4E-BP1
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Proteasome

Figure 3

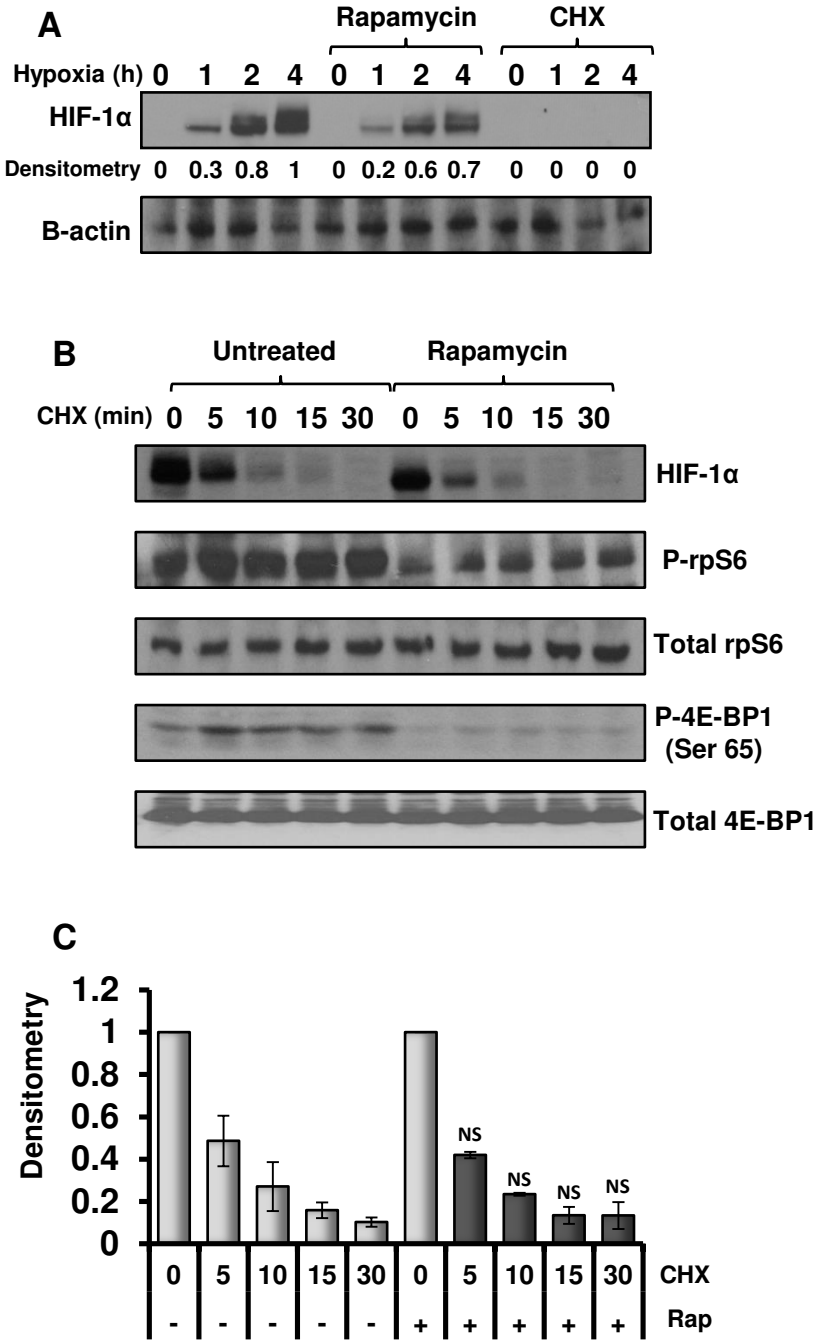
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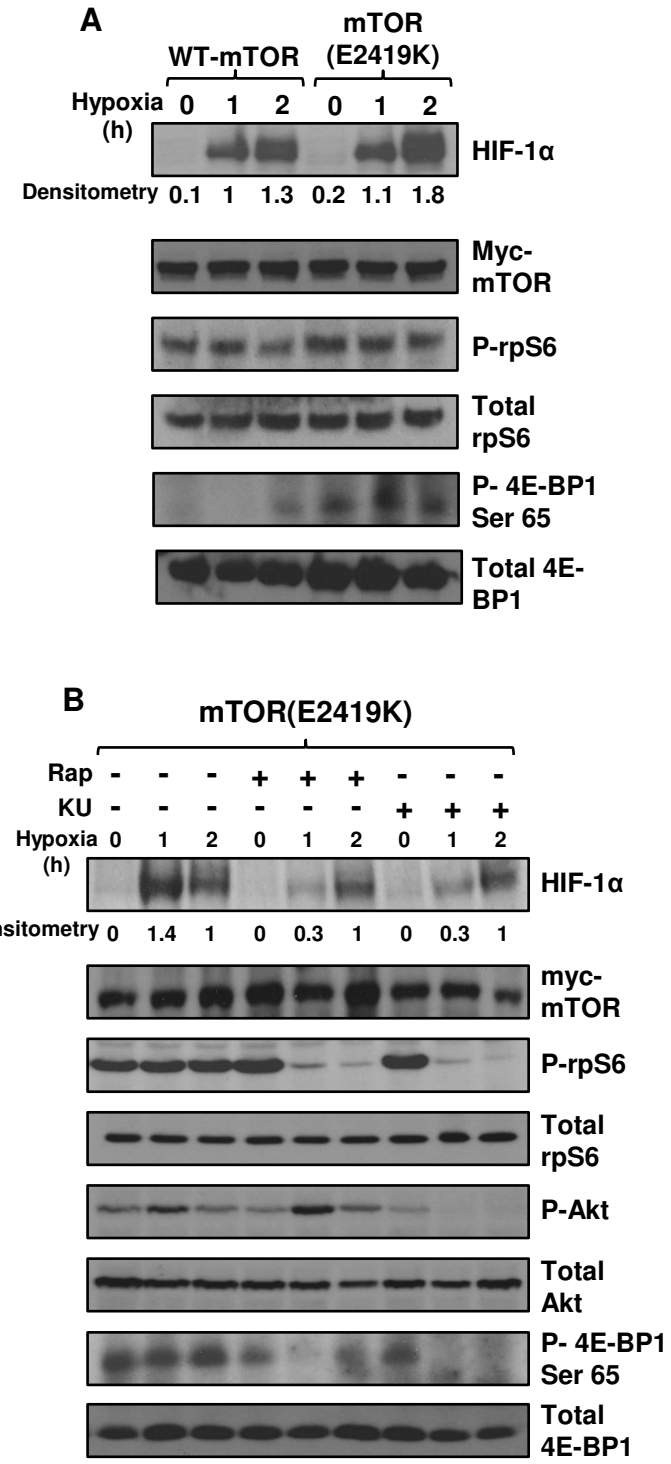
Figure 4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P-Akt
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Total 4E-BP1

Figure 5

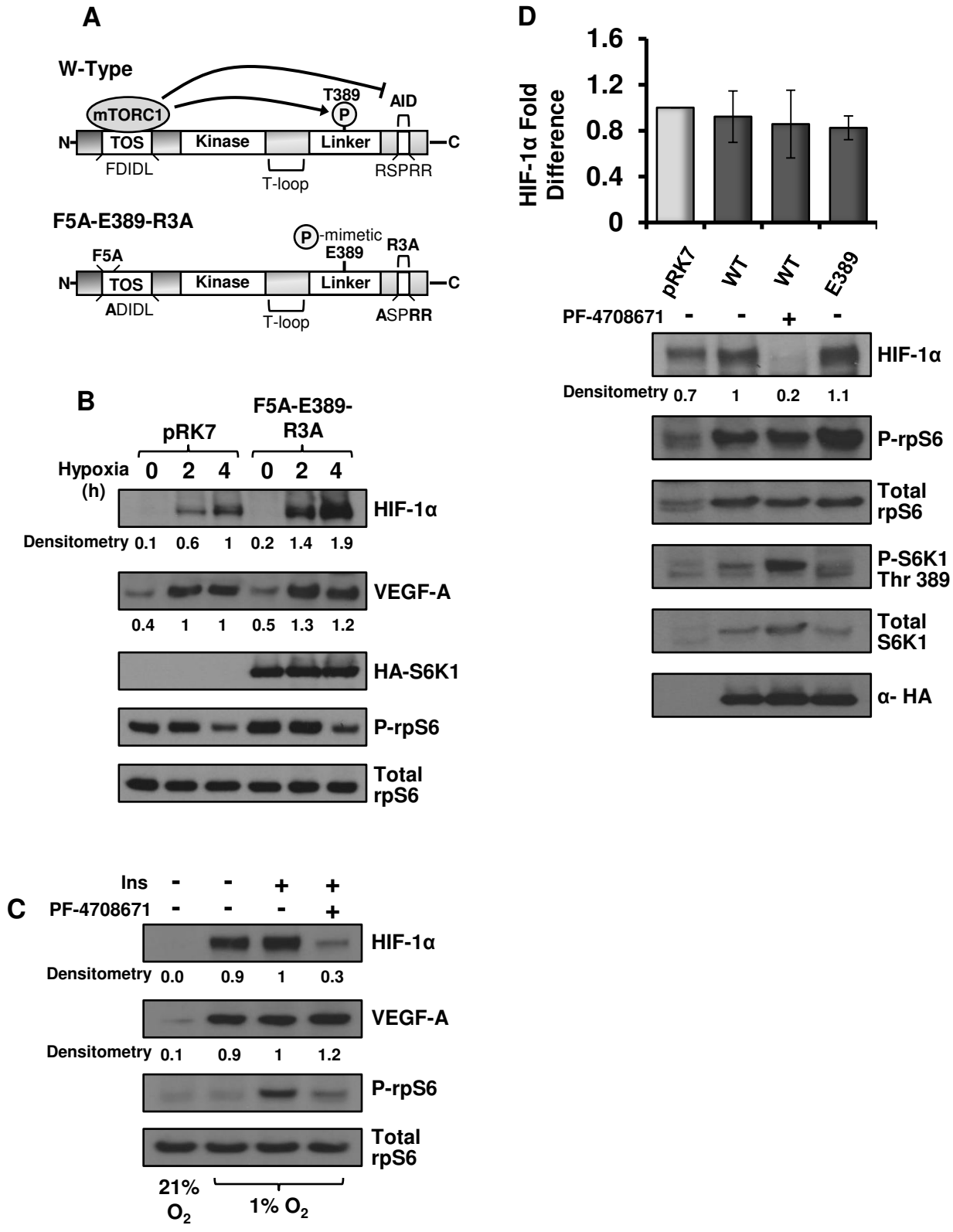

A
W-Type
mTORC1
T389
P
AID
N
TOS
Kinase
Linker
C
FDIDL
T-loop
RSPRR
F5A-E389-R3A
P-mimetic
E389
R3A
F5A
N
TOS
Kinase
Linker
C
ADIDL
T-loop
ASPRR
B
pRK7
F5A-E389-R3A
Hypoxia (h)
0 2 4 0 2 4
HIF-1α
Densitometry 0.1 0.6 1 0.2 1.4 1.9
VEGF-A
0.4 1 1 0.5 1.3 1.2
HA-S6K1
P-rpS6
Total rpS6
C
Ins - - + +
PF-4708671 - - - +
HIF-1α
Densitometry 0.0 0.9 1 0.3
VEGF-A
Densitometry 0.1 0.9 1 1.2
P-rpS6
Total rpS6
21% O2
1% O2
D
HIF-1α Fold Difference
1.6
1.2
0.8
0.4
0
pRK7
WT
WT
E389
PF-4708671 - - + -
HIF-1α
Densitometry 0.7 1 0.2 1.1
P-rpS6
Total rpS6
P-S6K1 Thr 389
Total S6K1
α- HA

Figure 6

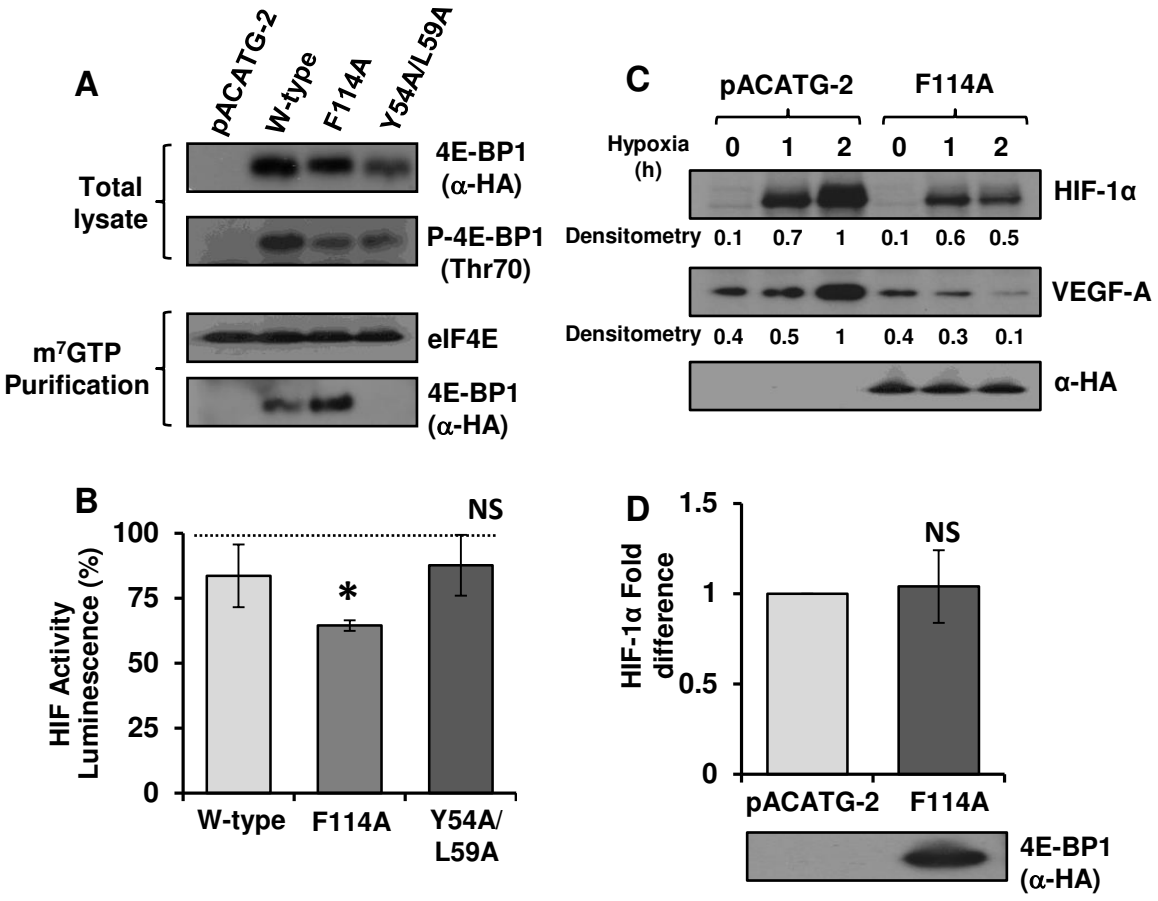

A
pACATG-2
W-type
F114A
Y54A/L59A
Total lysate
4E-BP1 (α-HA)
P-4E-BP1 (Thr70)
m7GTP Purification
eIF4E
4E-BP1 (α-HA)
B
HIF Activity Luminescence (%)
100
75
50
25
0
NS
*
W-type
F114A
Y54A/ L59A
C
pACATG-2
F114A
Hypoxia (h)
0 1 2 0 1 2
HIF-1α
Densitometry 0.1 0.7 1 0.1 0.6 0.5
VEGF-A
Densitometry 0.4 0.5 1 0.4 0.3 0.1
α-HA
D
HIF-1α Fold difference
1.5
1
0.5
0
NS
pACATG-2
F114A
4E-BP1 (α-HA)

Figure 7

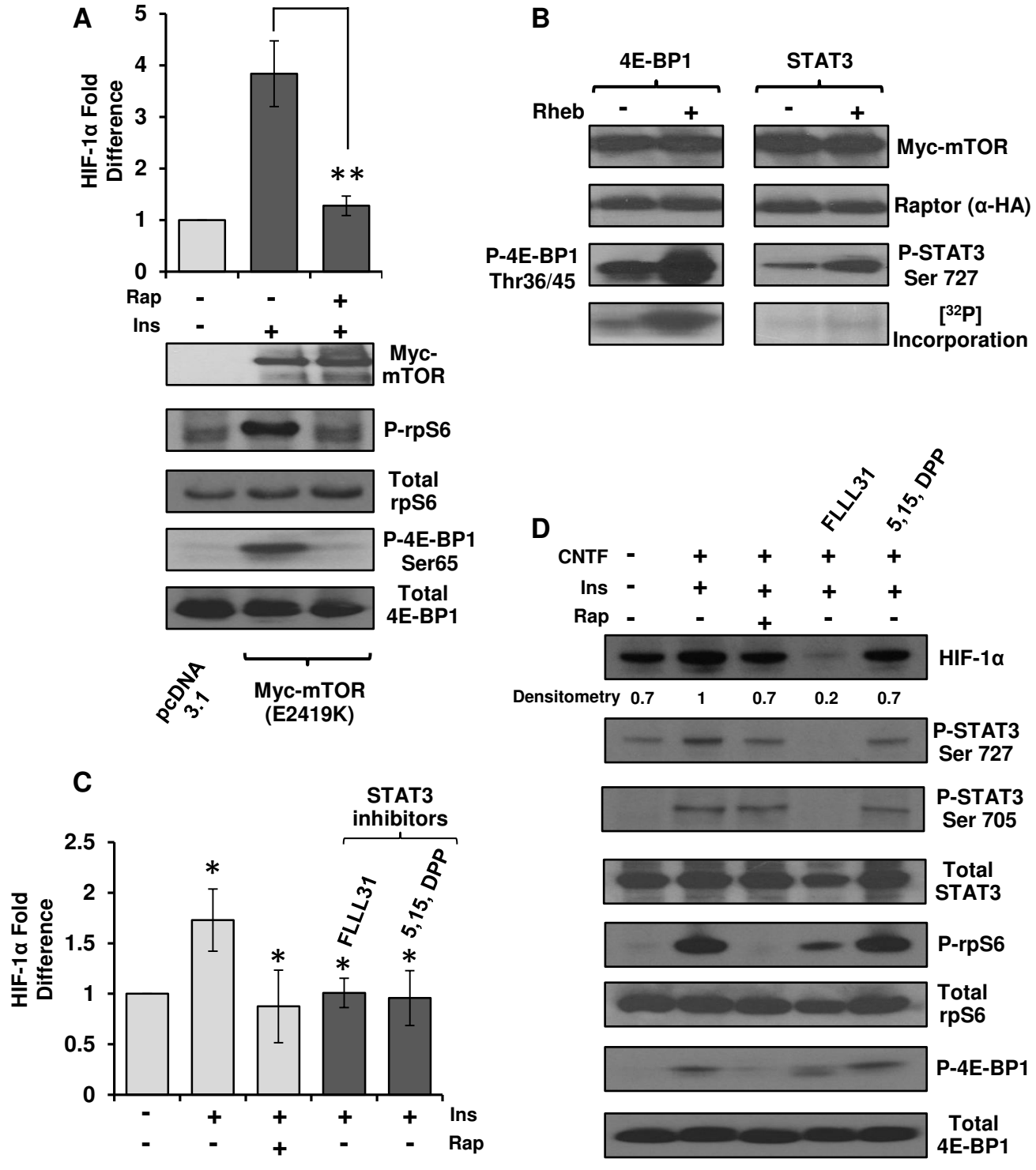
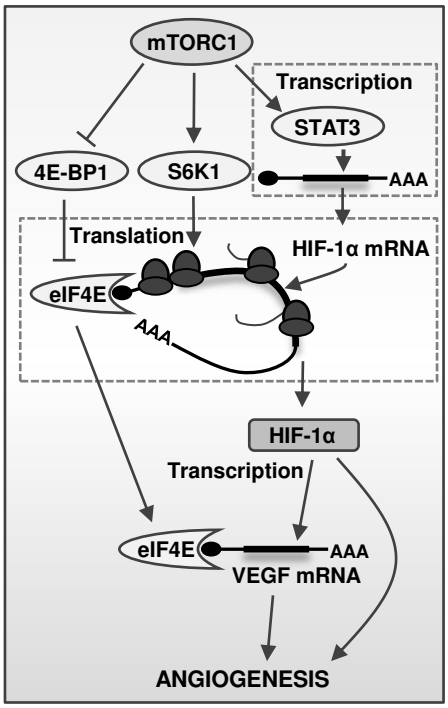

A
HIF-1α Fold Difference
5
4
3
2
1
0
**
Rap - - +
Ins - + +
Myc-mTOR
P-rpS6
Total rpS6
P-4E-BP1 Ser65
Total 4E-BP1
pcDNA 3.1
Myc-mTOR (E2419K)
B
4E-BP1
STAT3
Rheb - + - +
Myc-mTOR
Raptor (α-HA)
P-4E-BP1 Thr36/45
P-STAT3 Ser 727
[32P] Incorporation
C
STAT3 inhibitors
HIF-1α Fold Difference
2.5
2
1.5
1
0.5
0
*
*
* FLLL31
* 5,15, DPP
- + + + + Ins
- - + - - Rap
D
FLLL31
5,15, DPP
CNTF - + + + +
Ins - + + + +
Rap - - + - -
HIF-1α
Densitometry 0.7 1 0.7 0.2 0.7
P-STAT3 Ser 727
P-STAT3 Ser 705
Total STAT3
P-rpS6
Total rpS6
P-4E-BP1
Total 4E-BP1

Figure 8


mTORC1
Transcription
STAT3
4E-BP1
S6K1
AAA
Translation
HIF-1α mRNA
eIF4E
AAA
HIF-1α
Transcription
eIF4E
AAA
VEGF mRNA
ANGIOGENESIS